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THE IN VIVO LOCALIZATION
OF STAPHYLOCOCCAL ENTEROTOXIN B

✓ B

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Staphylococcal enterotoxin B is a low molecular weight protein exotoxin capable of producing acute staphylococcal food poisoning in experimental animals.(1) In addition, the purified toxin injected intravenously into rabbits and monkeys produces lethargy, fever, shock and death (2) while the same toxin given to rats and mice appears to have little clinical effect. Since there is no evidence of humoral immunity, the reasons for this difference in species susceptibility as well as the mechanism of action of the toxin itself remain unknown.

Although staphylococcal enterotoxin B (SEB) produces death in some laboratory animals, the clinical course as well as the pathology shows little to explain the cause of death. Apart from emesis, pyrexia, and hypotension of unknown etiology, clinical manifestations include mild hypoglycemia, elevation of blood urea nitrogen, thrombocytopenia, and transient leukopenia.(2-4) At necropsy the principal lesion is pulmonary interstitial edema, (5) but its occurrence is variable and not necessarily present in all animals.

Since neither morbid pathology nor the clinical course suggest a mechanism for the toxemia, we have been concerned with the fate of enterotoxin within the body in an attempt to locate and define the cellular sites of toxin action. It is the purpose of the present communication to describe means to identify the toxin in cells, as well as to measure its removal rate from blood. By combining fluorescent labeling of the toxin with fluorescent antibody methods, we have been able to correlate the vascular clearance of the toxin with its cellular localization in both rats and monkeys. Such studies have shown that the proximal convoluted tubules of the kidney are the principal sites of toxin removal and, further, suggest that the toxin gains access to these tubules by a process of glomerular filtration and tubular reabsorption.

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Enterotoxemia was induced in rats and monkeys by intravenous injection of the purified SEB. The biologic distribution of the unlabeled toxin was then assessed by fluorescent antibody methods one hour after challenge (Table I). No significant differences in distribution were observed at this time period between the two animal species for the tissues examined. In both species, a minimal positive reaction was observed in spleen and gastrointestinal tract; whereas, the lung and multiple sections at various levels of the brain were negative. The liver of the monkey had occasional but infrequent fluorescence whereas the liver of the rat failed to show significant localization. In contrast to these organs, the kidneys of both species exhibited a brilliant green luminescence. The location of this fluorescence appeared to be confined to the proximal convoluted tubules as little fluorescence was observed in distal tubules and glomeruli were consistently negative.

The results of two other methods for evaluating SEB distribution in the intact animal are also summarized in Table I. When the toxin was labeled with the fluorescent reagent fluoresceine isothiocyanate (SEB-FIT), its distribution could be observed directly in tissue sections examined in ultraviolet light. However, there were no significant differences in distribution between the labeled toxin and that of unlabeled toxin identified by the direct fluorescent antibody technique. On the other hand, radioactive iodinated toxin did show some preference for liver which was not appreciated with use of the other two methods. All methods indicated a high concentration of toxin in the kidney.

Although labeling of the toxin with either a fluorochrome or radioactive iodine did not destroy its toxicity, the labels did have an effect on the rate of clearance of the toxin from the circulation (Table II). Thus, when SEB labeled with radioactive iodine was injected into rats its rate of vascular clearance was significantly faster than the clearance of SEB-FIT. Since the rate of clearance was proportional to the toxin concentration, the clearance rate constant or K was obtained from the slope of the plot of the natural logarithm of the toxin concentration against time. Following bilateral nephrectomy, there was a dramatic retardation in the clearance rate of SEB but the clearance remained exponential. This reduction in clearance rate was used to calculate the amount of toxin removed by the kidney, since the difference in clearance rates before and after nephrectomy represented the rate of toxin accumulation by the kidney. At 60 minutes after injection between 75 and 80% of the toxin regardless of label in both rats and monkeys theoretically should have been cleared by the kidney. This observation underscored the importance of the kidney in the removal of intravenously injected toxin and confirmed the impression of the distribution of SEB as indicated by fluorescence microscopy and reported in the previous table.

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The question next arose as to the mechanism of renal clearance. Therefore, the following sets of experiments were performed in order to determine whether the SEB localized in the renal tubules by a process of glomerular filtration and tubular reabsorption or by pinocytosis from the efferent glomerular blood.

The objective of the first set of experiments was to determine the earliest localization of the toxin in the renal tubules and to correlate its subsequent distribution with the time after challenge. It was reasoned that if the kidney samples were taken at sufficiently close intervals after challenge it might be possible to observe (a) whether the toxin was initially present within the tubular lumen and (b) the direction of migration of toxin across the cell. The experiments were done with both unlabeled SEB detected by fluorescent antibody methods and with SEB conjugated with fluoresceine isothiocyanate (FIT). Both methods gave essentially similar results.

The earliest time interval in which the toxin could be visualized in the renal tubules was 15 seconds after intravenous challenge. At this time the toxin was clearly present on the luminal side of the proximal convoluted tubules as a distinct thin layer of fluorescence adjacent to the cell surface. In some sections, it was possible to observe that the toxin was present at the glomerular tubular junction. However, it was confined to the tubular epithelium and was not observed adherent to the parietal layer of Bowman's capsule nor to glomerular capillary endothelium. Indeed, the toxin appeared to show an affinity only for the brush border of tubular epithelium. From 15 seconds to one minute, the intensity of the fluorescence increased, but its distribution relative to the cell did not change. Figure 1 is a representative section taken at one minute after challenge showing the toxin confined to the tubular lumen.

Five minutes after injection, the toxin was still located predominantly on the luminal surface of the tubular cell, but the fluorescence intensity had increased. By 15 minutes, a definite gradient of fluorescent intensity from the luminal side of the cell towards the base of the cell was observed. At 30 minutes the entire tubular cell was fluorescent (Figures 2, 3). In all sections examined the presence of fluorescence was confined to the proximal tubules and did not involve the glomerulus or the distal segments.

It was concluded that the earliest appearance of either unlabeled SEB or SEB labeled with FIT was on the luminal side of the tubular cell presumably adherent to the brush border of the proximal convoluted tubules. The SEB then migrated from the luminal side to the basal portions of the cell so that by 30 minutes the entire cell was fluorescent. The above findings strongly indicated that SEB was filtered by the glomerulus and reabsorbed by the proximal tubules. If this were so, then it ought to be possible

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to prevent the localization of SEB in the kidney by a change in tubular pressure. Such a change can be induced by ureteral ligation. For this experiment, a unilateral ligation was performed so that the opposite kidney could serve as a control. SEB-FIT was then administered at 6, 12 and 24 hours after ligation and tissue was obtained 30 minutes later.

In kidneys obtained at 6 hours after ureteral ligation, the ligated and control kidneys showed an equal intensity of fluorescence. However, by 12 hours there was a marked reduction in fluorescence intensity in those kidneys whose ureter had been ligated. This change was more pronounced at 24 hours (Figures 4, 5), at which time most of the renal cortex was devoid of identifiable SEB. No appreciable pathologic changes were evident by light microscopy. Thus, a change in tubular hydrostatic pressure could prevent the localization of SEB in the kidneys.

If SEB were filtered by the glomerulus, then some relationship should exist between the rate of clearance of SEB and the rate of glomerular filtration. The calculation of glomerular filtration rate was made assuming that all SEB reaching the glomerulus was filtered and that SEB was not removed by the kidneys in any other manner. Under these conditions glomerular filtration rate was equal to the product of the clearance rate constant of the kidney (i.e., the difference in rate constants before and after nephrectomy) and the animal's blood volume. Table III presents the results. It is observed that in both rat and monkey there is a remarkable similarity between the accepted value for glomerular filtration rate and that derived from SEB clearance.

From the foregoing, it is proposed that the toxin gains access to the renal tubules by a process of glomerular filtration and tubular reabsorption. The evidence which supports this conclusion is the following: (a) the earliest appearance of the toxin following intravenous challenge is on the luminal surface of the proximal tubules; (b) in subsequent time periods, the toxin appears to move from the luminal side of the cell to involve the entire cell; (c) ureteral ligation, which produces a gradual increase in tubular hydrostatic pressure ultimately stopping glomerular filtration, prevents SEB from appearing in the kidney; and, (d) the rate of SEB clearance by the kidney is compatible with the rate of glomerular filtration.

The low molecular weight of the toxin (35,300) would be consistent with glomerular filtration. However, Crawley and co-workers, (2) who investigated the blood binding properties of SEB by precipitation methods, reported that 90% of intravenously injected SEB was transported in blood as a complex with serum albumin. From the study presented here, however, it now appears to be firmly established that intravenously administered SEB is rapidly removed from the circulation with concomitant accumulation

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in proximal convoluted tubules. Since it would be difficult to equate rapid vascular clearance with glomerular filtration - if indeed the toxin were bound to a circulatory transport protein - the next set of experiments was initiated to re-examine the question of whether staphylococcal enterotoxin exhibited any affinity for blood protein.

Immunoelectrophoresis was performed using both rat and monkey plasma and results were similar. A typical pattern for the rat is presented in Figure 6. Electrophoresis of SEB in plasma (100 µg/ml mixed at 37° C. for 30 minutes) was first performed and compared with an equal concentration of SEB in saline. In both instances, only a single precipitin band was found to rabbit anti-SEB and it was apparent that plasma had not altered the mobility of the identified fraction of SEB. Since on serial dilution, the SEB band in plasma disappeared at approximately the same dilution as SEB in saline (approximately 25 µg/ml) it was unlikely that any appreciable SEB complex had been formed in plasma whose immunological identity had not been detected. In order to observe whether the location of the SEB coincided with a plasma protein component, immunoelectrophoresis of plasma containing SEB was performed against rabbit anti-rat plasma and in the other direction against rabbit anti-SEB. As seen in Figure 6, there was no identity between the SEB band and any of the identifiable components of plasma. A similar result was obtained regardless of whether SEB was mixed with rat plasma in vitro or added to plasma by intravenous challenge (blood sample obtained 4 minutes after injection of 500 µg SEB/100 gm. body weight). Although the addition of SEB in monkey plasma in vitro (100 µg/ml) yielded a pattern similar to that of the rat, the concentration of SEB 4 minutes after intravenous injection in the monkey (1000 µg/kg) was not sufficient to resolve clearly the location of SEB by immunoelectrophoresis. However, the presence of SEB could be detected by Ouchterlony gel diffusion by using a larger sample volume.

Analysis by Ouchterlony gel diffusion failed to reveal any evidence of association between SEB and plasma proteins. A typical experiment using monkey plasma is presented in Figure 7. Only a single precipitin line was observed between plasma SEB and rabbit anti-SEB and this line was continuous with the precipitate formed with SEB in saline. The fact that the precipitin line appeared at the same distance between SEB in saline and anti-SEB as between SEB in plasma and anti-SEB, when equal concentrations of SEB were used, indicated that the coefficient of diffusion of SEB alone and SEB in plasma was identical. Furthermore, when the plasma components were identified with goat anti-monkey, it was apparent that the SEB precipitin line extended completely across the lines of all major plasma components.

Finally, since the molecular weight of SEB at 35,300 is less than that of most plasma proteins, it was possible to separate SEB from plasma using molecular sieve chromatography. When SEB alone was applied to a Sephadex G-75 column, it was eluted in a volume significantly greater than that of the protein peak of plasma applied alone. Therefore, it would be expected that when plasma and SEB were present together and if SEB were bound to a blood protein, it should migrate at a faster rate and appear in the plasma protein peak. However, when the SEB was added to plasma either by injection in vivo or by incubation in vitro, there was no significant change in the elution volume of SEB. Similar results were obtained with both rat and monkey plasma.

Whereas failure to identify a complex by immunologic methods might occur if the character of the SEB were altered by complex formation, this possibility was excluded by the chromatographic experiments and by the fact that on serial dilution all SEB in plasma could be accounted for as a single band on immunoelectrophoresis. Despite the inability to clearly locate SEB on immunoelectrophoresis pattern from the monkey following intravenous challenge, it could be identified on Sephadex G-75 and Ouchterlony gel diffusion. These experiments, together with observations made when SEB was added to monkey plasma in vitro support the contention that the behavior of SEB in monkey plasma is similar to that of rat plasma. Therefore, the present studies demonstrate the unlikelihood of association between SEB and the plasma proteins of a nonimmune animal and imply the restriction that if such binding does occur it must be of weak character and easily dissociable. This conclusion implies that the behavior of the toxin in the circulation and its biologic fate following intravenous injection probably reflect the intrinsic properties of the SEB molecule itself.

Although the majority of the toxin is rapidly removed from the circulation by the kidney, it was important to establish not only the duration of renal toxin localization but also whether sites other than the kidney might become involved late in the course of toxemia. Accordingly, the distribution of fluorescein-labeled toxin was examined in the hours following challenge in both monkeys and rats. Table IV presents the findings.

In the normal rat the toxin remained in the kidneys without apparent diminution of fluorescence intensity for 4 to 8 hours postchallenge. Thereupon a decrease was observed so that by 12 hours visible fluorescence was half that observed at one hour. Nevertheless, fluorescence was still present at 24 hours. The only site other than the kidney that concentrated sufficient toxin for visualization was the liver at 2 to 4 hours postchallenge. The importance of the liver can be demonstrated further by preventing the toxin from gaining access to the kidneys. Thus, in the nephrectomized rat, the liver fluorescence was intensified

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revealing it to be a site of toxin concentration and an alternative site to the kidney. The majority of the toxin was confined to Kupffer cells. This observation was compatible with data obtained using radio-iodinated labeled toxin in which it was shown that the highest concentration of toxin in the nephrectomized rat occurred in the liver although significant radioactivity also appeared in the lung, skin, and gastrointestinal tract. When the monkey was examined instead of the rat a major difference appeared in the duration of the toxin localization in the kidney as may be seen in Table IV. While considerable toxin was present in the rat kidney at 8 hours postinjection, it was effectively gone from the monkey kidney in the same time interval.

In summary, the present experiments based on morphologic examination and clearance kinetics clearly indicate that the kidney is the predominant site of toxin localization after intravenous injection of (SEB). Furthermore, our experiments show that the majority of the toxin found in the kidneys of both rat and monkey is located in the proximal convoluted tubules. Evidence has been advanced to substantiate the hypothesis that toxin gains access to the renal tubules by glomerular filtration and tubular reabsorption. Having gained access to proximal tubules, the toxin remains there for several hours. The possibility that SEB recycles through the kidney by being transported across renal cells and returned to the circulation is not excluded by the present experiments. However, significant redistribution of labeled toxin from the kidney to other sites did not appear to occur despite the increased fluorescence noted with time in liver Kupffer cells as well as a slight increase in radioactively labeled toxin in the lung. Of considerable interest is the fact that the alternative site to toxin localization in the kidney is the liver and that at no time could evidence be obtained for cerebral localization of the toxin. The absence of toxin in the brain is consistent with previous findings that there is no electro-encephalographic alteration in toxemic animals (6) and that the initiation of vomiting depends on local stimulation of a reflex arc originating in the gastrointestinal tract.(7) It is concluded that the kidney may play a major role in the pathogenesis of enterotoxemia.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences -- National Research Council.

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TABLE I
Biologic Distribution of SEB One Hour After Challenge.

		Tissue	Method	Kidney	Liver	Lung	Spleen	GI	Brain
Agent	1	F1. Ab.	+	-	-	±	±	±	-
SEB	Rat		++++	±	-	-	-	-	-
SEB-FIT	Rat	F1. Micros.	++++	±	-	+	+	±	-
		Counts % Dose							
SEB- 125	Rat		56.7	5.4	0.68	1.22	4.49	0.03	
SEB	Monkey	F1. Ab.	++++	±	-	±	±	?	?
SEB-FIT	Monkey	F1. Micros.	++++	±	-	±	±	±	-
		Counts % Dose							
SEB- 131 (Ref. 13)	Monkey		42	25	5	0.15	6	0.2	

Abbreviations: Staphylococcal enterotoxin B (SEB); fluoresceine isothiocyanate labeled toxin (SEB-FIT); radioactive iodine tagged toxin (SEB-125I or SEB-131I); fluorescence (F1); antibody (Ab); microscopy (micros.). Distribution by radioactivity is expressed as per cent of injected dose counted in the entire organ.

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TABLE II. Clearance Data and Toxin Distribution in Kidney (see text). The clearance rate constant is represented by K.

		Intact Animal	Post Nephrectomy	Theoretical % Injected Dose Kidney
	<u>Animal</u>	K	K	
SEB-FIT	Rat	0.082	0.015	81.8
SEB- ¹²⁵ I	Rat	0.121	0.031	74.3
SEB- ¹²⁵ I	Monkey	0.096	0.022	76.8

TABLE III. Calculation of Glomerular Filtration Rate (GFR)

Toxin	Animal	Kidney Clearance Constant	Blood Volume	SEB	GFR Accepted
SEB-FIT	Rat	.067	6.2 ml/100g	0.42	0.60 (8,9)
SEB- ¹²⁵ I	Rat	.090	6.2 ml/100g	0.56	0.60 (8,9)
SEB- ¹²⁵ I	Monkey	.074	44.7 ml/kg	3.31	3.66 (10)

TABLE IV. Extent of Fluorescence by Time After Injection of SER-FIT

		5 min	30 min	1 hr	2 hr	4 hr	8 hr	12 hr	24 hr
Normal Rat	Kidney	++	++++	++++	+++	+++	++	++	±
	Liver	-	-	±	+	+	±	±	-
Nephrecto- mized Rat	Liver	±	+	+	++	++	++	++	±
	Kidney	++	++++	++++	+++	+++	++	++	-
Normal Monkey	Liver	-	-	-	-	-	-	-	-

Sections of lung, brain, and heart were devoid of specific fluorescence at the time periods examined in both rat and monkey. Questionable fluorescence was observed on occasion in spleen and gastrointestinal tract. No definite localization of toxin was found for the nephrectomized monkey.

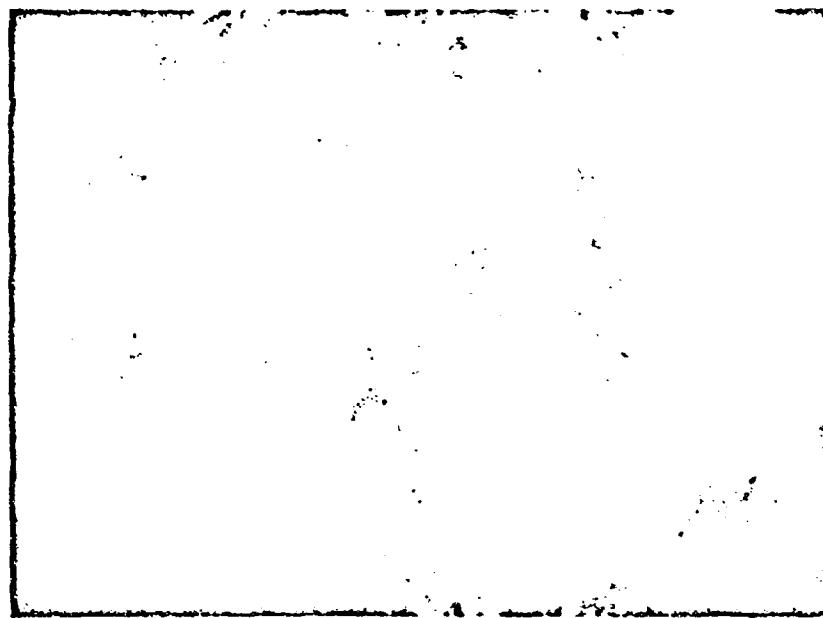


Fig. 1. Localization of SEB in rat kidney 1 minute after injection.
Fluorescent antibody method. Fluorescence remains on the
luminal border of the cell. X 820



Fig. 2. Localization of SEB in rat kidney 30 minutes after in-
jection. Fluorescent antibody method. Fluorescence is
now seen throughout the cell. X 1440

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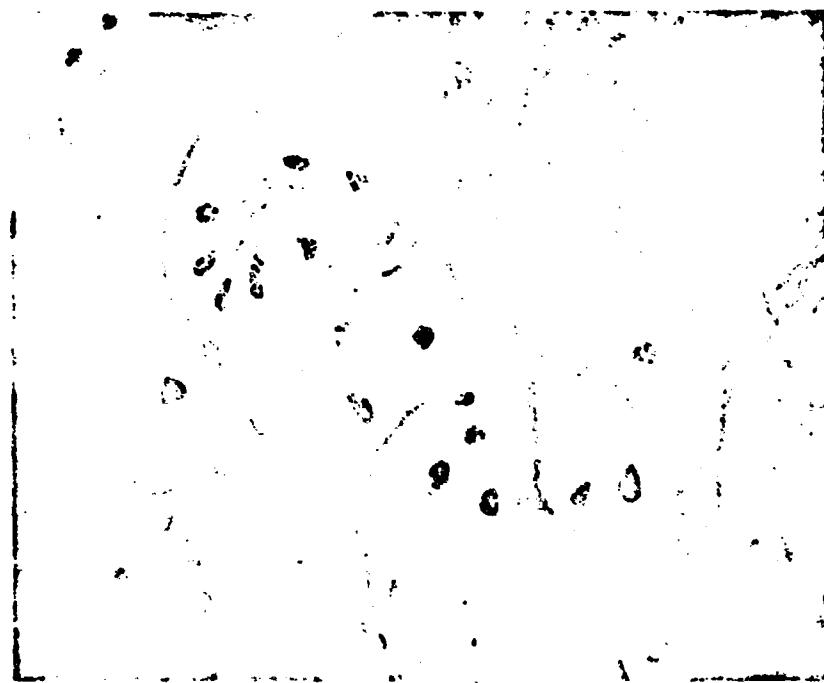


Fig. 3. Localization of SEB in monkey kidney 60 minutes after injection. Fluorescent antibody method. This picture shows tubular fluorescence adjacent to a glomerulus which is devoid of fluorescence. X 1440

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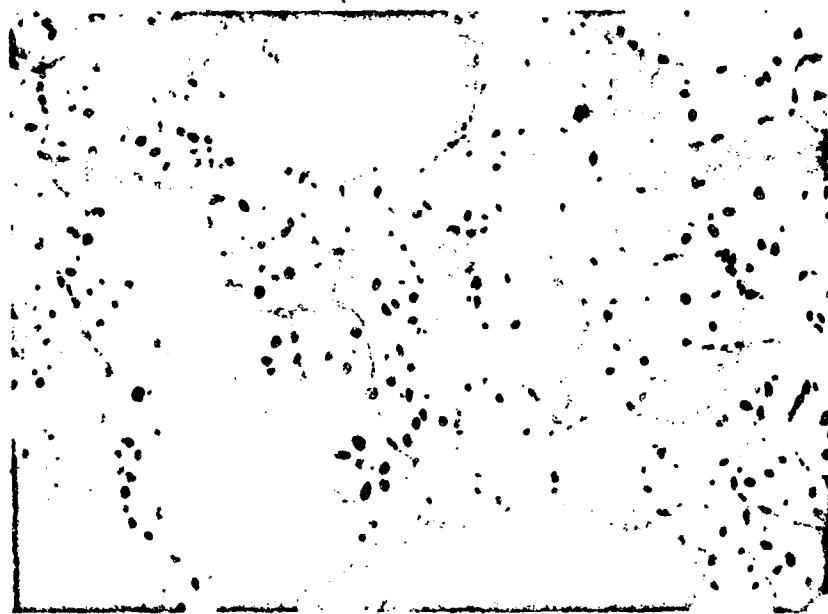


Fig. 4. Localization of SEB in rat kidney 30 minutes after injection. SEB-FIT with fluorescence microscopy. This kidney is from the control side of a rat whose opposite kidney had its ureter ligated. Compare this picture with Fig. 5.
X 520

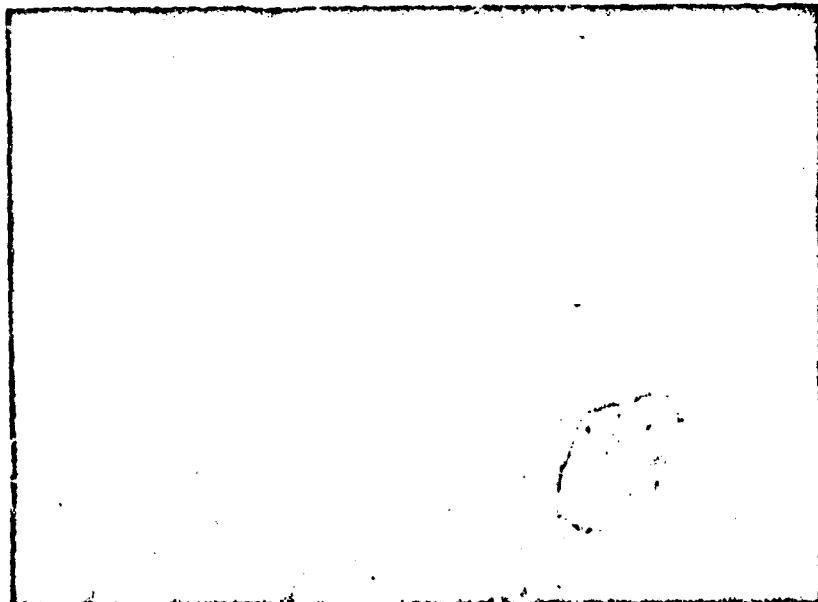


Fig. 5. Localization of SEB in rat kidney 30 minutes after injection with ureter ligation 24 hours prior to injection. SEB-FIT with fluorescence microscopy. Note the marked decrease in fluorescence and in the number of tubules involved. X 520

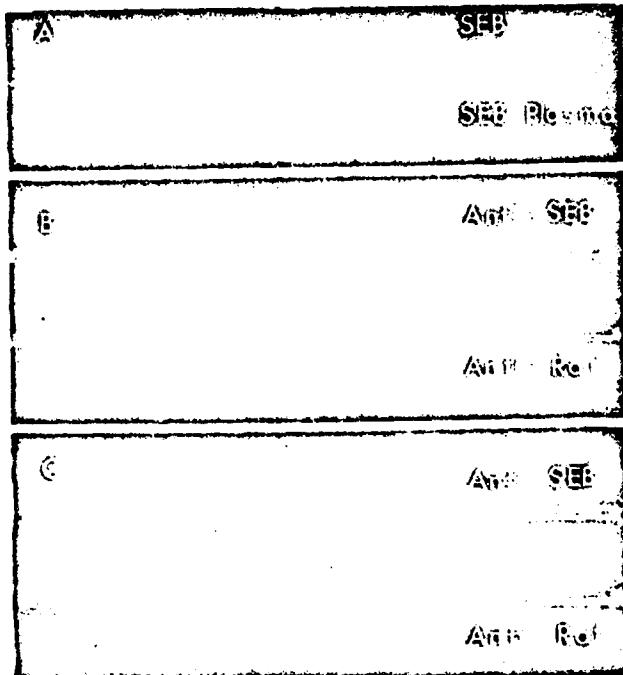


Fig. 6. Immunoelectrophoresis of staphylococcal enterotoxin B (SEB) and plasma proteins. The anode is on the left. Electrophoresis for 45 min at 12 ma per strip, using 0.02 M phosphate buffer, pH 7.4, with sodium chloride added to bring ionic strength to 0.100. Experiment A shows location of SEB in rat plasma identical to that of SEB in saline (immunodiffusion against rabbit anti-SEB). In experiment B, the electrophoretic sample was SEB-incubated with plasma in vitro; in Experiment C, the sample was obtained from a rat 4 min after SEB injection. In both experiments, the location of the SEB band is identical to that shown in Experiment A, and the SEB band is not associated with any identified plasma protein.

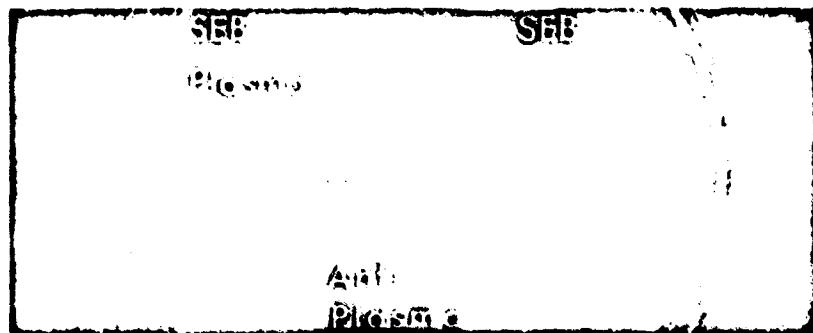


Fig. 7. Ouchterlony gel-diffusion analysis of staphylococcal enterotoxin B (SEB). The center well is rabbit anti-SEB. The other wells contain SEB in saline, SEB in monkey plasma, and goat antimonkey sera.